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Short communication

Diversity in *Encephalartos woodii* collections based on Random Amplified DNA markers (RAPD's) and Inter-Specific Sequence Repeats (ISSR's)

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Abstract

Variations in morphological characters inspired us to subject various genotypes of *Encephalartos woodii* to DNA based marker analyses. Genomic DNA of five genotypes of *E. woodii* growing in the environs of Durban, South Africa was amplified using 15 RAPD and 5 ISSR primers. The amplification profiles of both marker systems resulted in differences in the detected banding patterns. The dendrogram generated using the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) grouped all the *E. woodii* genotypes in one group. The two genotypes of *E. natalensis* analyzed for comparison were clustered in a different group. Due to different hierarchical positions in the dendrogram it is clear that the genome of all *E. woodii* accessions is not exactly the same. The reasons for such variations could well be that the Ngoye and Krantzkloof types originally found in two very distinct parts of Natal did not originate from the same parent(s).

Keywords: Cycads; Genetic similarity; Phylogeny; Polymerase chain reaction

1. Introduction

Encephalartos woodii Sander is one of the rarest plants in the World. It no longer exists in the wild. A single male multi-stemmed plant was found in the Ngoye forest in 1895 by Medley Wood. An offset of the main trunk of this specimen was planted in Durban Botanical Garden in 1903 (Osborne, 1986). Despite numerous extensive collecting trips no female plants have ever been found. Fortunately the male plant produce offsets and it appears that presently there are over 500 clones of the original plant in various parts of the World, either in private collections, cycad refuges or Botanical Gardens (Jones, 1993; Osborne, 1995). The origin of this species remains enigmatic. One view is that it is a hybrid between *E. natalensis* and *E. ferox* while another speculates that it evolved by mutation from *E. natalensis* Dyer & Verdoorn (www.plantzafrica.com/plantefg/encephwoodii.htm). Despite speculation that *E. woodii* has *E. natalensis* as a parent, comparison of the chloroplast DNA of the two species using restriction fragment length polymorphic (RFLP) analysis, the two species were reported to be distinct (Moretti and Norstog, 1992). A recent study using RAPD's indicated a close relationship between *E. woodii* and *E. natalensis* (Viljoen and Van Staden, 2006). It was suggested that *E. woodii* may well be a product of hybridization.

An interesting, yet not so well known fact, is the discovery of another single, male, multi-stemmed plant, very similar to the Ngoye *E. woodii*, closer to Durban near the present Krantzkloof Nature Reserve in the Umgeni River Valley. Most of this plant, including the main trunk, was transplanted into a private garden in the Kloof area (Grobbelaar, 2007). This plant is referred to as the Krantzkloof *E. woodii*. The leaves from mature specimens of the two *woodii's* and *E. natalensis* were previously compared (Osborne and Baijnath, 1995). The conclusion was that the Krantzkloof *E. woodii* is intermediate between the Ngoye *E. woodii* and *E. natalensis*. The authors speculated "that the

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Table 1
Sequences of the RAPD and ISSR primers used in the present study

Sequence no	Primers	Sequences
ISSR		
1	ISSR-1	5′-AGAGAGAGAGAGAGAGT-3′
2	ISSR-2	5′-GAGAGAGAGAGAGAGAT-3′
3	ISSR-3	5′-AGAGAGAGAGAGAGAGYC-3′
4	ISSR-4	5′-GAGAGAGAGAGAGAGAYT-3′
5	ISSR-5	5′-ACACACACACACACACYG-3′
RAPD		
6	OPB-01	5′-GTTTCGCTCC-3′
7	OPB-07	5′-GGTGACGCAG-3′
8	OPB-09	5′-TGGGGGACTC-3′
9	OPB-15	5′-GGAGGGTGTT-3′
10	OPC-02	5′-GTGAGGCGTC-3′
11	OPC-08	5′-TGGACCGGTG-3′
12	OPC-12	5′-TGTCATCCCC-3′
13	OPD-05	5′-TGAGCGGACA-3′
14	OPD-11	5′-AGCGCCATTG-3′
15	OPD-17	5′-TTTCCCACGG-3′
16	OPD-18	5′GAGAGCCAAC-3′
17	OPE-01	5′-CCCAAGGTCC-3′
18	OPE-07	5′-AGATGCAGCC-3′
19	OPE-11	5′-GAGTCTCAGG-3′
20	OPE-17	5′-CTACTGCCGT-3′

Krantzkloof plants either represent a second population of *E. woodii* or that these plants had experienced a significant genetic contribution from that species". Recently the sporophylls of Krantzkloof and Ngoye *E. woodii* specimens were investigated. The male sporophyll morphology between the two so-called *E. woodii* clones differed considerably (Grobbelaar, 2007). The author raised the question whether the observed differences in reproductive structures warrants the allocation of different scientific names for the specimens. This question is indeed very intriguing and we applied molecular techniques in an attempt to throw further light on the genetic variability between the two *woodii* accessions.

2. Materials and methods

Young leaves of two Ngoye specimens (N1 (SP-93) and N2 (SP-111)), two Krantzkloof specimens (K1 (SP-112) and K2 (SP-127)) all growing in the same garden in Kloof and the original Ngoye *E. woodii* growing in the Durban Botanical Garden (DBG) (SP-73) were collected. For comparison young leaves of *E. natalensis* 1 (SP-29) were collected from the Durban Botanical Garden (DBG), while a second specimen, *E. natalensis* 2 (SP-116) was collected from the same garden from where N1, N2, K1 and K2 types of *E. woodii* were growing in Kloof. Material was transferred to the laboratory on dry ice and stored at −70 °C until analyses. Voucher numbers are indicated in brackets. All voucher specimens are housed in the University of KwaZulu-Natal Herbarium (NU) in Pietermaritzburg.

Genomic DNA was isolated from the stored leaves according to the protocol described by Rogers and Bendich (1988) with minor modifications. Briefly, liquid nitrogen was used to grind 500 mg of freeze-dried tissue. The powder was suspended in 3 ml CTAB extraction buffer [2% CTAB (Cetyl trimethyl ammonium bromide), 100 mM Tris–HCl (pH 8.0), 20 mM EDTA and 1.4 M NaCl] in 15 ml tubes. The suspension was incubated at 65 °C for 1 h and extracted with an equal volume of chloroform:iso-amyl alcohol (24:1) followed by centrifugation at 10,000 *g* for 10 min. The aqueous phase was transferred to a fresh tube and one-tenth volume of CTAB and NaCl (10% CTAB and 0.7 M NaCl) added. This was again extracted with an equal volume of chloroform:iso-amyl alcohol (24:1) and centrifuged as before. DNA was precipitated with 2–3 ml of iso-propanol for 20 min at −20 °C. The DNA recovered after centrifugation was dissolved in high salt TE [10 mM Tris–HCl (pH 8.0): 0.1 mM EDTA (pH 8.0) and 1 M NaCl] and extracted with equal volumes of phenol: chloroform:iso-amyl alcohol (25:24:1). After centrifugation, the aqueous phase was collected and DNA was precipitated with 1/10 volume of 5 M NaCl and three volumes of absolute alcohol for 10–20 min at −20 °C. The DNA recovered after centrifugation was washed three times with 70% alcohol followed by a single

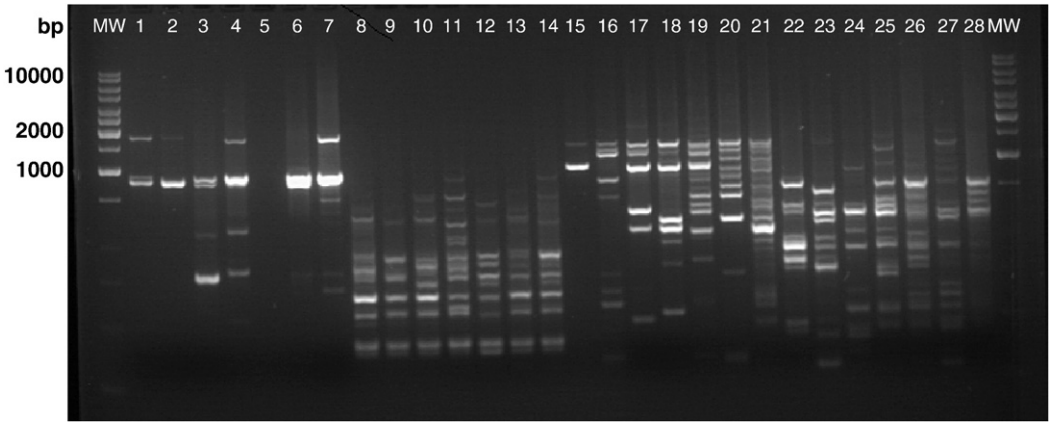

Fig. 1. DNA amplification profile of five genotypes of *Encephalartos woodii* and two genotypes of *E. natalensis* with RAPD primers. OPB-01 (Lanes 1–7), OPC-02 (Lanes 8–14), OPC-08 (Lanes 15–21) and OPD-18 (Lanes 22–28). *E. woodii* N1 (Lanes 1, 8, 15, 22); *E. woodii* N2 (Lanes 2, 9, 16, 23); *E. woodii* K1 (Lanes 3, 10, 17, 24), *E. woodii* K2 (Lanes 4, 11, 18, 25), *E. woodii* DBG (Lanes 5, 12, 19, 26), *E. natalensis* 1 (Lanes 6, 13, 20, 27); *E. natalensis* 2 (Lanes 7, 14, 21, 28).

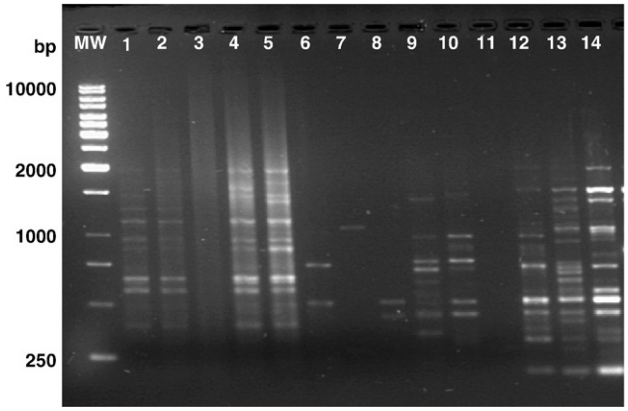

Fig. 2. Amplification DNA profile of five genotypes of *Encephalartos woodii* and two genotypes of *E. natalensis* with ISSR primers. ISSR-1 (Lanes 1–7), ISSR-4 (Lanes 8–14). *E. woodii* N1 (Lanes 1, 8); *E. woodii* N2 (Lanes 2, 9); *E. woodii* K1 (Lanes 3, 10), *E. woodii* K2 (Lanes 4, 11), *E. woodii* DBG (Lanes 5, 12), *E. natalensis* 1 (Lanes 6, 13); *E. natalensis* 2 (Lanes 7, 14).

wash in absolute alcohol. The DNA was finally dissolved in 20 ml HPLC water. The DNA was quantified by means of UV spectrotometery. The DNA pellet was dissolved in sterile HPLC water and diluted to a working solution of 10 ng/µl for PCR analysis. Five ISSR and 15 RAPD primers were used for fingerprinting. Sequences of the primers are given in Table 1. The amplification reaction was carried out in a volume of 25 µl containing 50 ng DNA, 3 µl of 10× PCR buffer (Bioline), 0.2 mM dNTP's (Bioline), 4 µM primer and 1 U Taq DNA polymerase (Bioline). Amplifications were carried out in DC-960G Gradient Thermal Cycler (Corbett Research, Australia). The PCR programme for ISSR primers was: initial denaturation at 94 °C for 5 min, followed by 1 cycle each of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 90 s, with a decrease of annealing temperature by 2 °C in subsequent cycles, until the annealing temperature reached 42 °C, this was followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 42 °C for 45 s and extension at 72 °C for 90 s, with a final extension at 72 °C for 5 min. For RAPD primers the programme has an initial denaturation at 94 °C for 5 min, followed by 1 cycle of denaturation at 94 °C for 1 min, annealing at 36 °C for 20 s, extension at 72 °C for 2 min, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing at 35 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 5 min.

The amplified samples were separated by electrophoresis on 1.4% agarose gel in 1× TAE (Tris–Acetic acid–EDTA) buffer. DNA was visualized with ethidium bromide (0.5 µg/ml) under UV light. A 1 kb ladder (Fermantas, Hannover, USA) was used as a molecular size marker. Amplifications were performed many times (at least 4) independently to ensure reproducibility and consistency.

Data was subjected to similarity matrix and cluster analysis using the Numerical Taxonomy and Multivariant Analysis System program package for PC (NTSYS-pc version 2.11U) (Rohlf, 1988). Percentages of polymorphic bands were defined as the percentage of polymorphic bands amplified by a single primer to that of the total number of bands produced by the same primer. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) using Sequential Agglomerative, Hierarchical and Nested Cluster (SAHN) (Sneath and Sokal, 1973).

3. Results and discussion

DNA fingerprinting profiles of the five genotypes of *E. woodii* exhibited a high degree of polymorphism with both types of DNA markers. Figs. 1 and 2 represent the sample gels showing the polymorphism existing among genotypes of *E. woodii* after amplification with RAPD primers (OPB-01, OPC-02, OPC-08 and OPD-18) and ISSR primers (ISSR-1 and ISSR 4). With RAPD primers, a total of 134 bands were scored out of which 110 (82.1%) were polymorphic, while 24 (17.9%) were

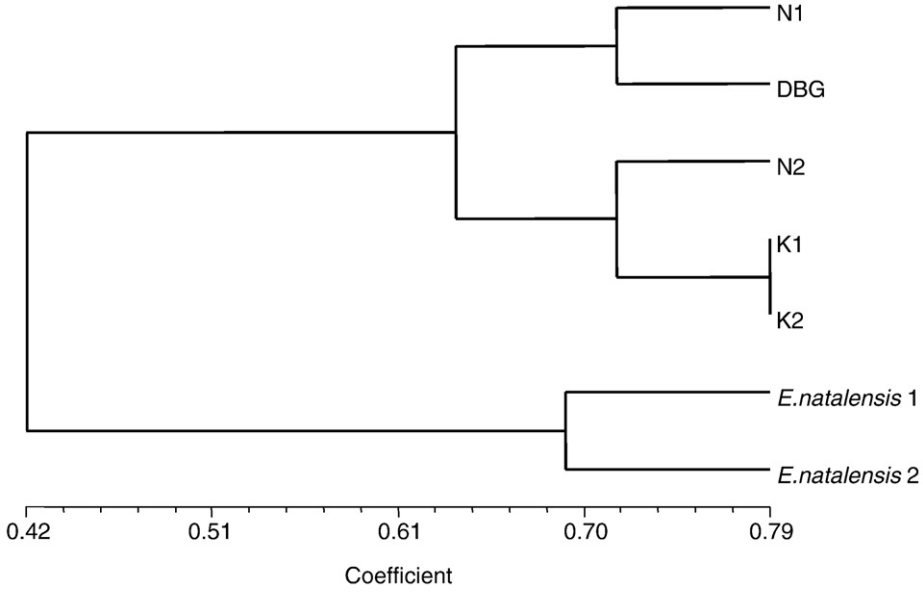

Fig. 3. Dendrogram generated by applying the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) to the data gathered using RAPD and ISSR primers.

monomorphic. However, with ISSR primers out of a total of 110 bands scored 86 (78.2%) were polymorphic and 24 (21.8%) were monomorphic. Similarity index values ranged from 0.564 to 0.794, indicating that genotypes screened are closely related. Young leaves of *E. natalensis*, collected in the same garden where the *E. woodii* specimens (N1, N2, K1, K2) were growing, were used as an outgroup as it had been suggested that *E. woodii* had evolved from *E. natalensis*. The similarity index value of *E. natalensis* exhibited variations in numerical values with different genotypes of *E. woodii* screened (0.256 to 0.487). Within the *E. woodii* genotypes, K1 and K2 had the highest similarity index value of 0.794, while the least similarity (0.564), was between K1 and DBG, and N2 and DBG. The two genotypes of Ngoye had a similarity value of 0.692, the reason why they are placed in different subgroups. N1 had a higher similarity index value of 0.718 with DBG when compared to that of N2 and DBG (0.564). Therefore, N2 was placed with K1 and K2 in one subgroup, while N1 and DBG forms a second subgroup (Fig. 3). The similarity index values of the two genotypes of *E. natalensis* were 0.692, indicating that they are closely related. These numerical values indicate that phylogenetically *E. natalensis* is only distantly related to *E. woodii*. These two genotypes were therefore placed together in a separate group (Fig. 3).

In the literature, no study is available wherein different genotypes of *E. woodii* (from Ngoye and Krantzkloof) were compared to analyze the extent of similarity and/or dissimilarity between them. Some studies are available where the phylogeny of different species of *Encephalartos* has been compared (Van der Bank et al., 1998, 2001; Treutlein et al., 2004). The restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA of *E. woodii* and *E. natalensis* revealed that these are distantly related (Norstog and Nicholls, 1997).

The results presented herein reveal that various genotypes of *E. woodii*, growing in the Durban area, South Africa, exhibit variations in their DNA fingerprinting profiles in spite of frequently being considered to have been derived from one source. We agree with Grobbelaar (2007) that there is a strong case on both morphological and molecular grounds to further investigate the taxonomic status of the Ngoye and Krantzkloof accessions.

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